XLIII. VITAMIN C AND THE SUPRARENAL CORTEX.

II. LOSS OF POTENCY OF GUINEA-PIG SUPRARENALS IN SCURVY. WITH NOTES ON A METHOD FOR DETERMINING ANTISCORBUTIC ACTIVITY (HEXURONIC ACID) BY CHEMICAL MEANS.

By LESLIE JULIUS HARRIS and SURENDRA NATH RAY.

From the Nutritional Laboratory, University of Cambridge and Medical Research Council.

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In Part I [Harris and Ray, 1932] it was shown that ox suprarenals have a very high antiscorbutic activity, commensurate with their richness in hexuronic acid. The principal purpose of the present paper is to describe results already briefly alluded to in Part I, showing that the suprarenal gland of the normal guinea-pig has an antiscorbutic activity similar to that of the ox, and that this activity disappears with the development of scurvy. A method is also described for estimating hexuronic acid chemically, and it is shown that the hexuronic acid contents of orange juice, lemon juice and other sources accurately account for their observed antiscorbutic activity.

Experimental.

Harris's curative method [Harris, Mills and Innes, 1932; Harris and Ray, 1932] was used. The whole glands were fed in this case (instead of cortex only as in the experiments with ox suprarenals), with no apparent ill effects. The glands from normal and scorbutic guinea-pigs were fed at the two levels of 0·5 g. and 1·0 g., and the results were compared with those given by orange juice fed as standard at the two levels of 1·5 cc. and 3 cc., and with negative controls (basal diet only). In order to obtain a sufficient fresh daily supply of suprarenals from scorbutic guinea-pigs, large numbers of animals had to be placed on a scurvy-producing diet at daily intervals at a suitable length of time before the beginning of the assay. During the course of the determination, some of these animals, then suffering from scurvy, were killed daily and their suprarenals fed with as little delay as possible to the test animals. The suprarenals were prepared for feeding in the manner described in Part I. The same procedure was used for testing the suprarenals of normal guinea-pigs except that the diet of the latter was supplemented with cabbage (10 g. *per diem*).

Result. 0·5 g. of normal guinea-pig suprarenal was found to be approximately equivalent in antiscorbutic activity to 1·5 cc. of orange juice, and 1·0 g. of suprarenal to 3·0 cc. of orange juice. In the case of the suprarenals from scorbutic guinea-pigs, no antiscorbutic action could be detected even when fed at levels of 1 g. per day. Thus, animals receiving 1·0 g. or 0·5 g. of the scorbutic suprarenal

lost weight at the same rate as the negative controls (see Fig. 1); and no differences were apparent in the survival periods (Table I) or in the degree of severity of the scurvy, as determined at autopsy.

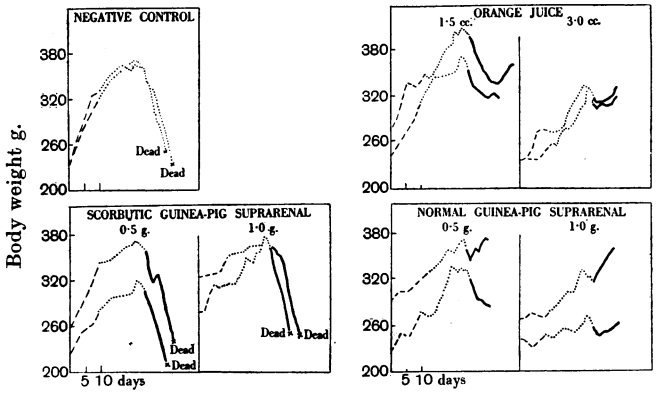

Fig. 1. – – – Preliminary period: scorbutic basal diet supplemented with cabbage.
. . . Depletion period: basal diet alone.
—— Experimental curative period.

Table I. *Survival periods, days (counting from beginning of depletion period).*

Negative controls	24, 22, 21	(av. 22·3)
Scorbutic suprarenal, 0·5 g. per day	24, 22	(av. 23)
Scorbutic suprarenal, 1·0 g. per day	26, 19	(av. 22·5)

Comments. The activity of the suprarenal of the guinea-pig is seen to be roughly three times as great as that of orange juice (wet weight). A similar value was found for the cortex of the suprarenal of the ox [Harris and Ray, 1932]. It follows therefore that weight for weight the whole suprarenal of the guinea-pig tends to be somewhat more active even than the whole suprarenal of the ox (in which species the medulla appears to be devoid of hexuronic acid).

Experiments are at present in progress to determine the antiscorbutic activity of the suprarenals of a number of dogs which have been maintained without ill effects for upwards of 7 months on a diet free from all vitamin C. Similar tests are being done on rats similarly kept for prolonged periods on vitamin C-free diets. Since these species are able to dispense with the need for vitamin C in their diet, it was of interest to compare their behaviour with that of guinea-pigs. Our colleague Dr Thomas Moore has examined the suprarenals for their hexuronic acid activity *in situ*, by the silver staining method of Szent-Györgyi [1928]. He has shown that whereas the suprarenals of normal guinea-pigs stain deeply with silver and lose this property after the animals have been kept for about 8 days on a scurvy-producing diet, the suprarenals of rats or dogs on the other hand continue to stain intensely even when there is no vitamin C in the diet [see Moore and Ray, 1932]. We have reached the same conclusion, determining the hexuronic acid chemically (see below).

Correlation between antiscorbutic activity and hexuronic acid content.

It was shown in Part I that the observed antiscorbutic activity of ox suprarenals could be accurately accounted for on the basis of their apparent hexuronic acid content. The relative antiscorbutic activities of ox suprarenals and orange

juice were proportional to the amount of hexuronic acid recoverable from the two sources. This was regarded as evidence in favour of the theory that hexuronic acid is identical with vitamin C. Additional arguments were also given in support of this view, but it was pointed out that further work would be needed before it could be regarded as definitely established that the activity of specimens of hexuronic acid was due to the main constituent and not to some undetected contaminant. A definite conclusion could only be reached by determining whether there existed a sufficiently constant and extended parallelism between antiscorbutic activity and hexuronic acid. One of the first desiderata to this end is to have a reliable method for estimating hexuronic acid chemically. We have carried out some experiments in this direction, as a result of which we have been able to show that the hexuronic acid content of orange juice, like that of suprarenals, is in accord with the theory that hexuronic acid and the vitamin are identical[1].

Prof. Szent-Györgyi suggested to us that the most hopeful method to try would be a rapidly carried out titration in acid solution either with iodine or dibromo-indophenol. The chief objection to the former method seemed to be that a considerable number of naturally occurring substances would reduce the iodine slowly, while glutathione would certainly reduce it rapidly, and hence the method would be inapplicable to the many natural sources containing glutathione. However, it gives theoretically perfect values with isolated hexuronic acid, and it seemed worth while to try it out, with various modifications, on certain sources, such as orange juice, known to contain little or no glutathione.

Unsatisfactory results with iodine titrations. Even with fresh orange juice, iodine titration in acid solution measures an appreciable amount of material in addition to hexuronic acid (judging the content of the latter from our later results), and it soon became apparent that the method was far too unspecific to give any reliable index. Orange juice which had been boiled and aerated to destroy the hexuronic acid and the antiscorbutic potency was found to show an

Table II. *Titration of specimens of fresh lemon or orange juice with standard iodine solution* (0·00895 *N*).

	Mg. of iodine reduced	Hexuronic acid equivalent, mg. per cc. of juice
Lemon juice, 5 cc.	3·94	
,, ...	3·93	
,, (in presence of 2 drops of glacial acetic acid)	3·91	0·54
,, (by back titration method, KI and $Na_2S_2O_3$)	3·75	
Orange juice, 5 cc.	6·36	
,, (in presence of 4 drops of glacial acetic acid)	6·30	0·87
,, (by back titration method, KI and $Na_2S_2O_3$)	6·05	

almost undiminished titre (Tables II and III). It would appear from this that while the hexuronic acid is oxidised, other reducing substances are simultaneously formed in the orange juice under these conditions [*cf.* Zilva, 1930]. Control tests

[1] Svirbely and Szent-Györgyi [1932] have already expressed the belief that "the hexuronic acid present in lemon juice has the same activity as the lemon juice itself," but they had no precise information either as to the minimum dose of hexuronic acid or the hexuronic acid content of lemon juice. The latter was assumed to be about 0·33 mg. per cc., or, in an earlier paper [Szent-Györgyi, 1928], 0·25 mg. per cc. (for orange juice)—*i.e.* about one-half the value we find in the present paper. Not more than 0·1 mg. has actually been isolated per cc., representing one-third to one-sixth of the amount thus calculated to be present.

with various reducing agents showed that a number of these do in fact readily reduce iodine in acid solution, including catechol, tannic acid, quinol, pyrogallol, protocatechuic acid and solutions of glucose or fructose which had been boiled for 2 hours. In the case of fresh lemon juice, iodine titration in acid solution gave an approximately accurate measure of the hexuronic acid content, judged by our later results.

Table III. *Iodine titration values of orange juice after boiling and aeration.*

	cc. of 0·00818 *N* iodine solution required by 5 cc. of the juice (at natural acidity of juice)
Orange juice, before treatment	5·1
,, boiled and aerated 30 mins.	5·6
,, ,, ,, 60 ,,	5·8
Orange juice, before treatment	5·0
,, aerated 30 mins.	4·6
,, ,, 60 ,,	4·5
,, ,, 95 ,,	5·15

Unsatisfactory results with 2:6-dichlorophenolindophenol in neutral solution. We next tried the use of this indicator in the manner described by Tillmans *et al.* [1932]. The procedure, in outline, is to titrate with the indicator until it is no longer reduced, the solution being first adjusted to near neutrality, by addition of sodium acetate, for which purpose the p_H colour change of the indicator itself is utilised. Here again the method broke down when tested by the titration values given by aerated orange juice (Tables IV and VIII); and it was found also that the indicator was reduced rapidly by pyrogallol or reduced glutathione and more slowly by catechol or tannic acid. The specificity of Tillmans' method therefore cannot be relied upon under certain conditions.

Table IV. *Titration of aerated orange juice with 2:6-dichlorophenolindophenol.*

	cc. of indicator solution required
5 cc. of orange juice, untreated (titrated at natural p_H of juice)	6·03
,, aerated for 40 mins. (titrated at natural p_H of juice)	5·67
,, ,, 60 ,, ,, ,,	5·7
,, ,, 100 ,, ,, ,,	5·7
2 cc. of orange juice, untreated (neutralised with 25 cc. 20 % Na acetate)	2·5
,, aerated for 60 mins. (neutralised with 25 cc. 20 % Na acetate)	2·5

Method adopted. A method depending on the use of the same indicator was eventually worked out which, when tested against various naturally occurring reducing agents, other than hexuronic acid (including pyrogallol, catechol, tannic acid, quinol, reduced glutathione and fresh and boiled solutions of glucose, fructose or sucrose[1]), was found to give consistently negative results, and which behaved satisfactorily with test solutions of hexuronic acid partially inactivated and with aerated fruit juice. There is little doubt that when applied for example to orange juice it can give results only little above the actual hexuronic acid content. The indicator is made up at a strength of about 0·01 *M*. To carry out a

[1] Pyrogallol and quinol reduce the indicator very slowly under these conditions, while with boiled sucrose or fructose solutions the colour is discharged on standing for some time: this behaviour is so different from that of hexuronic acid as not to affect the accuracy of the method.

titration the solution to be analysed is first brought to p_H about 2·5 (measured preferably by the hydrogen electrode) through the addition of a few drops of strong acetic acid. To a suitable volume of the solution (*e.g.* 5 cc. for orange juice) indicator is rapidly run in until it is no longer reduced. At first we experienced some difficulty in determining this somewhat indistinct end-point, especially when working with coloured solutions such as orange juice; but it was found that the difficulty could be entirely overcome by making use of some additional chlorophenolindophenol, used as external indicator, and a sharp end-point was reached. (The indicator is red, instead of blue as in Tillmans' method.) It has since been found that, for still greater precision, it is preferable to carry out the estimation by taking a measured volume of indicator solution and titrating it with the unknown [Birch, Harris and Ray, 1932]. To give the result in terms of mg. of hexuronic acid the indicator solution has to be standardised against a given solution of hexuronic acid, which in turn has to be standardised for its true hexuronic acid content. The last operation is readily carried out by means of an iodine titration, it being necessary only to remember that 1 molecule of hexuronic acid reduces 2 atoms of iodine. The procedure is best made clear by an actual instance. In the example cited (Table V) iodine titration showed that the

Table V. *Standardisation of hexuronic acid against iodine, and indicator solution against hexuronic acid.*

10 cc. of hexuronic acid solution (containing 1·72 mg. of a specimen of hexuronic acid) were titrated—

(1) against 0·00677 *N* iodine solution:
I required = 2·31 mg.;
Hence hexuronic acid present = 1·60 mg.;

(2) against indicator solution:
Volume of indicator required = 2·7 cc.
Hence, 2·7 cc. of indicator solution ≡ 1·6 mg. of hexuronic acid
or, 1 cc. ,, ≡ 0·6 mg. ,,

specimen of hexuronic acid used for making the test solution contained only 93 % of actual (fully reduced) hexuronic acid. Titration of this standardised hexuronic acid solution against the indicator solution now showed that 1 cc. of indicator solution was equivalent to 0·6 mg. of true hexuronic acid. Next titration of orange juice with this same indicator solution (Table VI) gave the result

Table VI. *Estimation of hexuronic acid in orange juice by titration with standardised indicator in acid solution.*

2·0 cc. of fresh orange juice, reduced to p_H 2·59, required of standardised indicator 2·05 cc.
Hence 2·0 cc. of orange juice contain 1·2 mg. of hexuronic acid.

that 2 cc. of orange juice reduced 2·0 cc. of indicator. Hence it was calculated that 2 cc. of orange juice contain 1·2 mg. of hexuronic acid. Table VII shows that this titration method does not give anomalous results with aerated specimens of orange juice, like the methods described earlier. With most natural sources of the vitamin a preliminary extraction process with trichloroacetic acid, sometimes followed by decoloration, is necessitated. With the numerous plant and animal sources so far examined (including orange juice, lemon juice, cabbage, watercress, grape-fruit, pineapple, tomato, horseradish, several varieties of apple, ox suprarenal, ox liver, cow's milk) the hexuronic acid content so determined agrees exactly with that calculated from the antiscorbutic activity. A typical result for lemon juice is set out in Table VII, and further results and a more detailed discussion will be given in a later paper [Birch, Harris and Ray, 1933].

Table VII. *Estimation of hexuronic acid content of lemon juice.*

1 cc. of hexuronic acid solution (about 0·1 %) required	1·2 cc. of indicator solution 2·57 cc. of 0·00453 *N* I solution.
Hence 1 cc. of indicator ≡	0·8 mg. of hexuronic acid
2 cc. of lemon juice, required (at p_H 2·5)	1·4 cc. of indicator solution.
Hence 2 cc. of lemon juice contain 1·1 mg. of hexuronic acid.	

Table VIII. *Control tests on aerated and heated orange juice, comparing Tillmans's with new method.*

	Cc. of indicator required	
	Tillmans' method	New method
2 cc. of orange juice, before treatment	2·1	2·0 (≡1·16 mg. hexuronic acid)
,, aerated for 2 hours	2·4	1·6
,, boiled for 2 hours	1·9	0·7

Table IX. *Further control tests with aerated orange juice,* etc., *(titrating in acid solution).*

	Cc. of indicator required (varying strengths were used in the different experiments)
5 cc. of orange juice, before treatment	8·0
,, made alkaline and aerated for 30 mins.	2·0
,, ,, ,, 60 ,,	1·1
,, ,, ,, 90 ,,	0·6
2 cc. of fresh orange juice (p_H reduced to 2·59)	2·05
,, +15 cc. of water	2·0
,, ,,	2·1
,, made alkaline and aerated for 2 hours	0·15
5 cc. of hexuronic acid solution (containing 1 mg. of original preparation)	
Before treatment	2·2
After aeration for 15 mins. in alkaline solution	0·2
,, 30 ,, ,,	0

Agreement between observed and predicted hexuronic acid content. 1 cc. of orange juice contains, according to our indicator titration results, 0·6 mg. of hexuronic acid or slightly under, or according to the iodine titration result less than 0·8 mg. (since the iodine reacts with a certain amount of additional material in fresh orange juice as well as the hexuronic acid). This value agrees well with that predicted on the basis of the relative antiscorbutic activities of hexuronic acid and orange juice, on the supposition that vitamin C is identical with hexuronic acid. Thus according to Harris, Mills and Innes's [1932] assay of hexuronic

Table X. *Hexuronic acid content of orange juice, as determined chemically and as calculated from antiscorbutic activities.*

Method	Hexuronic acid mg. per cc.
Determined by chlorophenolindophenol titration	0·6
Determined by iodine titration	<0·8
Calculated from curative tests	Between 0·4 and 0·66
Calculated from preventive tooth-structure tests	,, 0·33 and 0·66
Calculated from assumption that minimum preventive dose = 1·0 mg. to 0·5 mg.	,, 0·33 and 0·66
Probable value	0·5–0·6

acid by the curative method, 2 mg. of hexuronic acid were somewhat more potent than 3 cc. of orange juice but less potent than 5 cc. of orange juice. (Confirmatory results, using hexuronic acid from different sources and a highly repurified specimen are shown in Fig. 2.) That is, 1 cc. of orange juice is antiscorbutically equivalent to rather less than 0·66 mg. of hexuronic acid but more than 0·4 mg. Again, the minimum protective dose of hexuronic acid (*i.e.* corresponding with 3·0 cc. of orange juice) as determined by the microscopic tooth-structure method was greater than 1 mg. but less than 2 mg. [Harris, Mills and Innes, 1932] so that on this basis 1 cc. of orange juice is equivalent to between 0·33 and 0·66 mg. of hexuronic acid. Again Svirbely and Szent-Györgyi [1932] found that 1 mg. of hexuronic acid daily sufficed as a preventive (smaller amounts were not tried), and if this be regarded as the minimum daily protective dose (*i.e.* corresponding with 1·5 cc. of orange juice), then 1 cc. of orange juice is equivalent to 0·66 mg. of hexuronic acid. King and Waugh's [1932] claim to a minimum dose of 0·5 mg. is probably slightly underestimated and would lead to the rather lower value of 0·33 mg.; the true value is probably between the two.

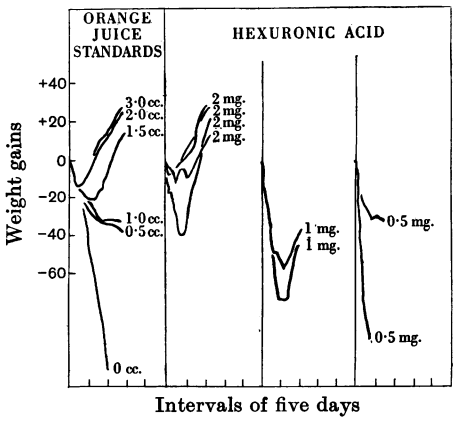

Fig. 2. Curative tests comparing hexuronic acid (weight curves of individual animals are shown) and orange juice (weight curves are averages of groups of four animals).

It may be concluded, then, that whatever slight adjustments in these values may be necessitated (as a result of more precise measurements of the activity of the acid, or the amount of it in orange juice) there can be no doubt that the antiscorbutic activity of orange juice agrees within very close limits with its hexuronic acid content (Table X). For lemon juice likewise (as for many other plant sources of vitamin C) we have found that indicator titrations lead to a similar conclusion. Moreover we have seen (Part I) that the same is true of suprarenal cortex; and, as our present results show, scurvy causes the disappearance of vitamin C from this organ, which coincides with a simultaneous loss of hexuronic acid. Again we have found that the destruction of hexuronic acid proceeds at a similar rate to that of its antiscorbutic activity. Investigations are also in progress in which antiscorbutic activity has been correlated with hexuronic acid content as measured by other indices as well as reducing reactions, and fuller details will be published later [Birch, Harris and Ray, 1933]. In conclusion therefore it may be claimed that an extensive quantitative correlation has already been proved between hexuronic acid and antiscorbutic activity.

SUMMARY.

The suprarenal of the normal guinea-pig has an antiscorbutic activity and hexuronic acid content similar to (or somewhat greater than) that of the ox. The activity disappears together with the hexuronic acid when vitamin C is withheld from the diet. A preliminary account is given of experiments contrasting this behaviour of the guinea-pig with that of other species such as the dog and rat, which can synthesise the vitamin when none is provided in their food.

A method of estimating hexuronic acid has been worked out, depending on modifications in the use of Tillmans's reduction indicator 2·6-dichlorophenol-indophenol. The hexuronic acid content of orange juice determined by this means accounts accurately for its observed antiscorbutic potency. A similar correlation has been established between hexuronic acid and vitamin C in the cases of lemon juice and various other sources, normal and scorbutic suprarenals, and during destruction by oxidation, *etc.*, and supports the theory of the identity of the two.

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